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(54) Enzyme immunoassay

(57) The present invention relates to the conventional solid-phase enzyme immunoassay, in which all the steps of from the step of immunological reaction of the immunologically reactive component(s) or an immunological complex thereof with a carrier-adsorbed reactant to the step of measuring the activity of enzyme contained in the resulting insolubilized product are carried out by applying chromatography with a single column.

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Fig. 1

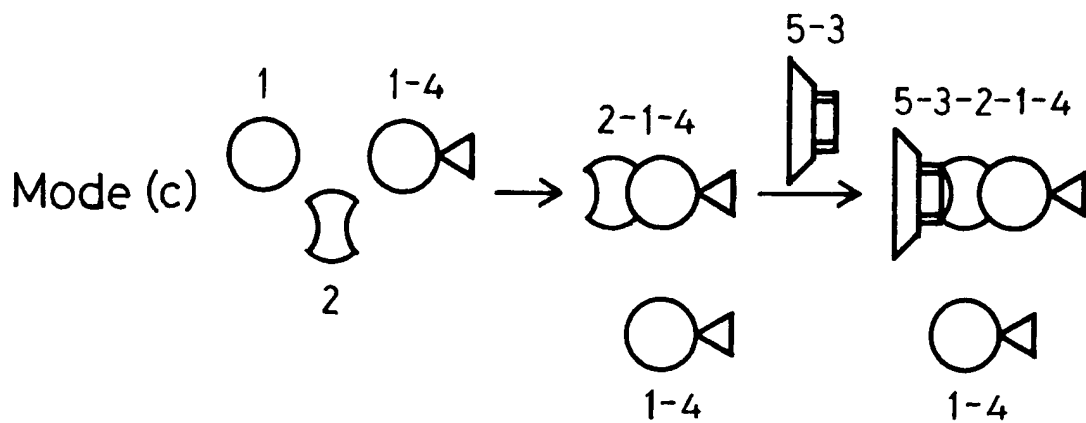
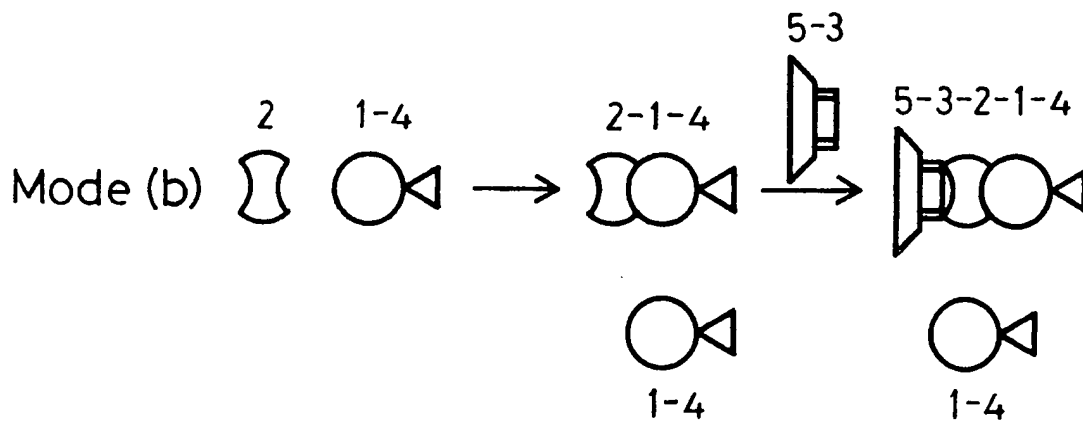
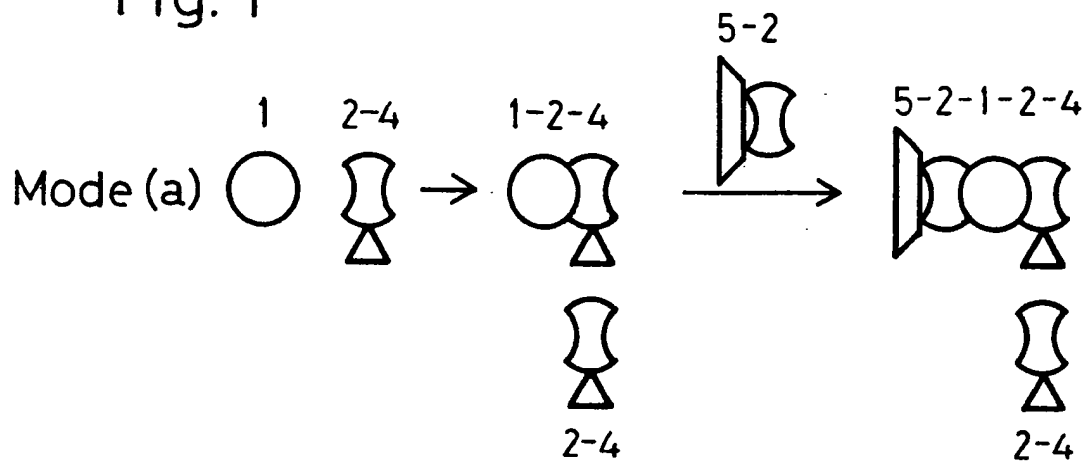


Fig. 1 (cont.)

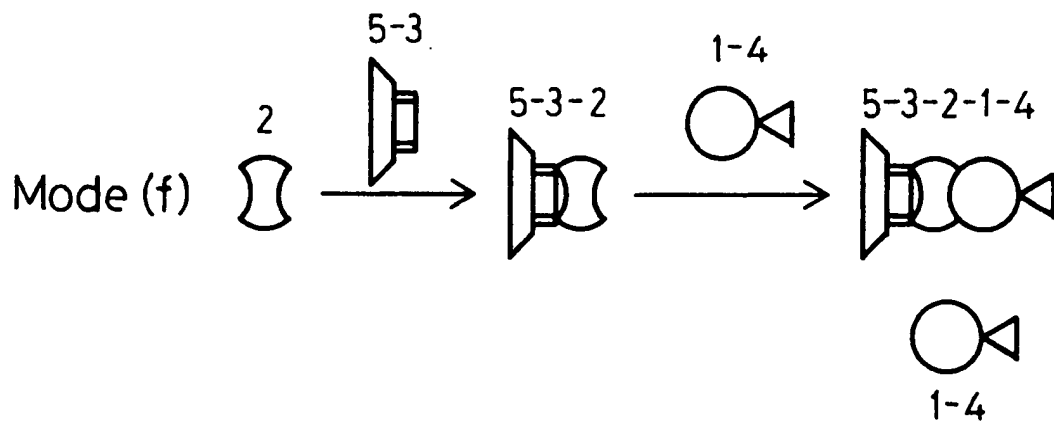
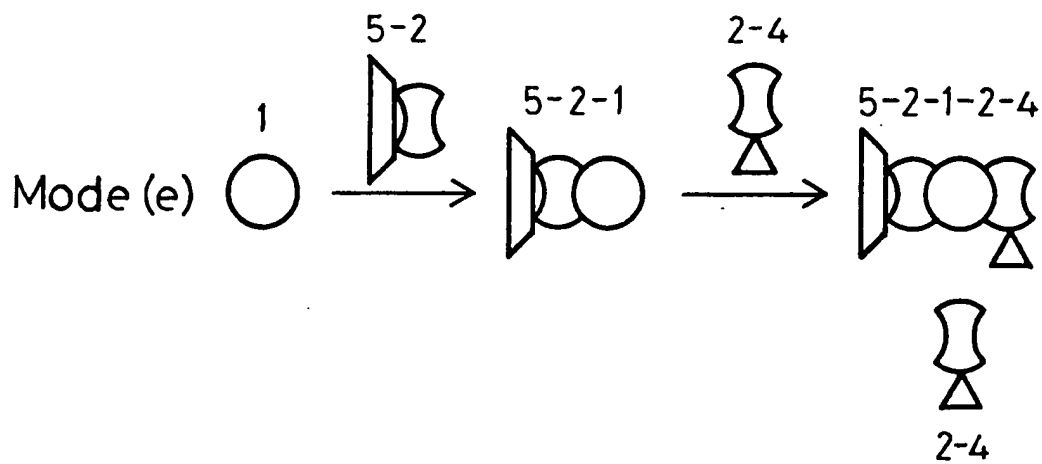
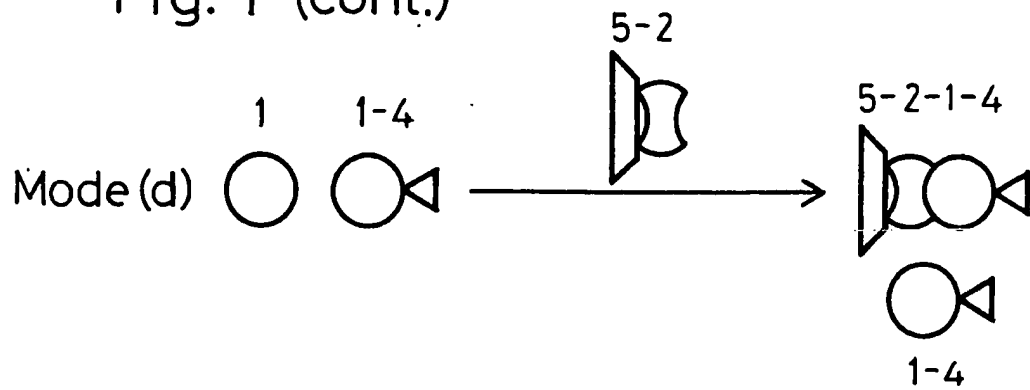


Fig. 2

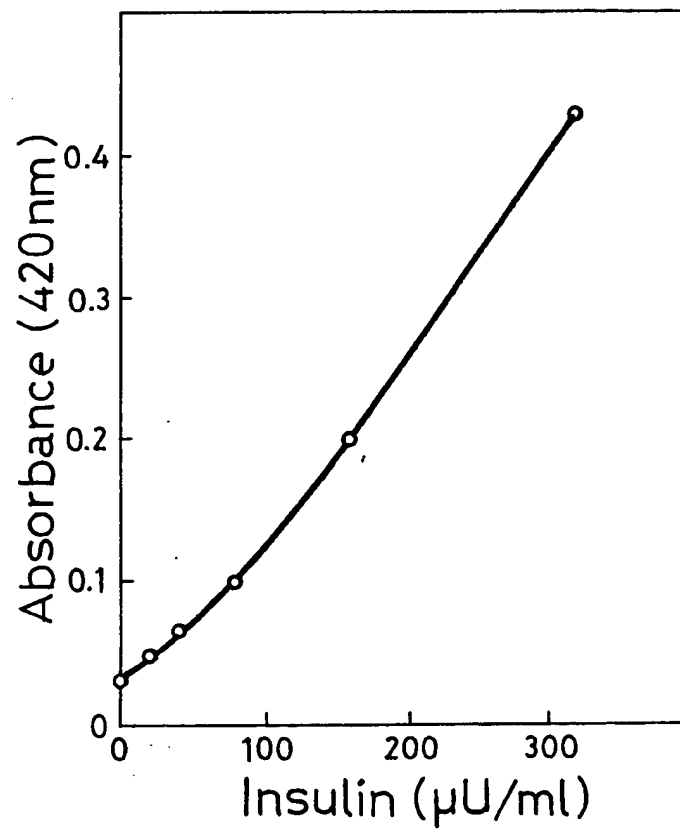
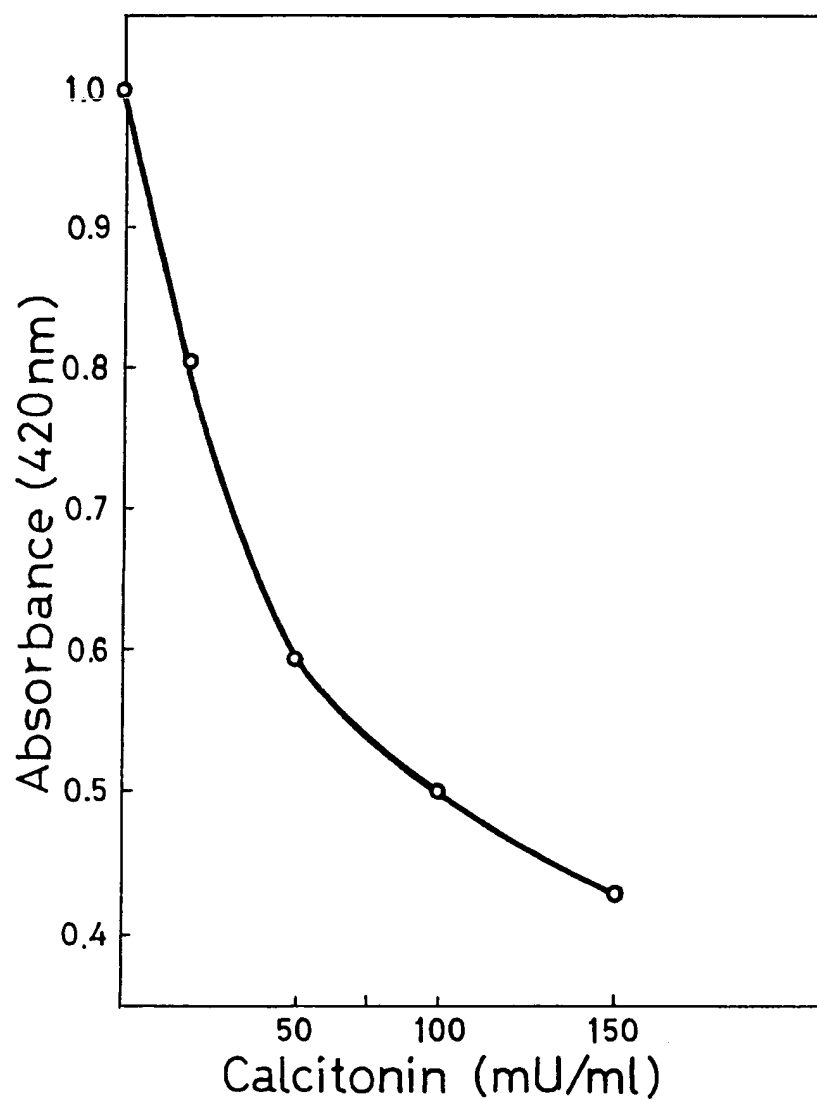


Fig. 3



## SPECIFICATION

### Enzyme immunoassay

#### 5 *Technical field*

This invention relates to a novel immunoassay method for the determination of amounts of infinitesimal substances present in body (or biological) fluids, such as serum and cerebrospinal fluid, of

10 mammals.

The term "immunoassay" as used herein refers to a method in which the reaction of an immunological-ly (serologically) antigenic substance (i.e., a so-called antigen) with a so-called antibody immuno-  
15 logically corresponding to it is applied to the determination of amount or concentration of antigen or antibody present in the fluid.

#### *Background art*

20 Conventionally, various methods such as bioassay or enzyme activity measurement methods have been available for the determination of amounts of infinitesimal substances present in biological fluids. However, they are not satisfactory because of their  
25 troublesome operations and their inadequate sensitivity or accuracy. In contrast, the immunoassay technique which has recently been developed can attain significantly high sensitivity and accuracy without requiring difficult operations. Accordingly,  
30 this technique is now being employed in the fields of biochemistry and clinical medicine.

Various kinds of immunoassay methods have been proposed, among which the methods using an antigen or antibody combined (or labeled) with a  
35 radioisotope, a fluorescent material or an enzyme have found wide application. Specifically further, the so-called solid-phase modes in these immunoassay methods using the labeling materials have been regarded as being most advantageous.

40 In the solid-phase immunoassay method, the so-called carrier-adsorbed reactant is used as a reaction material, which has been prepared by adsorbing, under specific reaction conditions, an antibody, a so-called anti-antibody (i.e., an antibody  
45 produced by using an antibody as the antigen), or so-called protein A (i.e., a special protein produced by certain microorganisms and having the ability to react with various antibodies) on a water-insoluble carrier such as pieces of polystyrene or silicone,  
50 water-insoluble polysaccharides, etc., to insolubilize the former thereby, said carrier-adsorbed reactants in particular cases being hereinafter referred to antibody-on-carrier, anti-antibody-on-carrier, or protein A-on-carrier, respectively.

55 Now the general principle of solid-phase immunoassay method using an enzyme as the labeling material is explained hereinbelow before proceeding to the description of the present invention.

At least one number selected from the group  
60 consisting of an antigen, an antibody, a labeled antigen, and a labeled antibody (these are preferred generally to immunologically reactive components hereinafter), or an immunological complex thereof, is reacted immunologically with a carrier-adsorbed  
65 reactant selected from the group consisting of an

antibody-on-carrier, an anti-antibody-on-carrier, and protein A-on-carrier at once or in twice, thereby  
70 result an insolubilized product formed by the binding of the labeled antigen (or labeled antibody) or its immunological complex to the carrier-adsorbed reactant together with an unbound, soluble portion of the labeled antigen (or labeled antibody). After the unbound labeled antigen (or labeled antibody) is separated from the insolubilized product, the activity  
75 of the enzyme contained in the insolubilized product (particularly, the absorbance of reaction mixture of the enzyme with its substrate) is measured. Typically, the same procedures as above are repeated with varying amounts of the antigen (or antibody), and by  
80 using the measured values thereby, a calibration curve is constructed which represents the relationship between the amounts of antigen (or antibody) initially used and the activity of enzyme attached thereto. Next, the same procedures are carried out  
85 with a biological fluid containing an unknown amount of the antigen (or antibody), to measure the activity of the enzyme contained in the resulting insolubilized product. Then, the amount or concentration of antigen (or antibody) present in the fluid is  
90 calculated from the measured value by reference to the calibration curve.

While the solid-phase enzyme immunoassay method as above has advantageously high sensitivity and accuracy, it has the disadvantage of requiring  
95 troublesome operations.

#### *Disclosure of the invention*

We studied the conventional solid-phase enzyme immunoassay method extensively and invented a  
100 very efficient technique thereof, by conducting all the steps from the step of the immunological reaction of immunologically reactive component(s) or an immunological complex thereof with a carrier-adsorbed reactant through to the step of measuring the activity of the enzyme contained in the resulting insolubilized product by applying chromatography with a single column.

According to the method of the present invention, a variety of antigens or antibodies can be assayed by  
110 using an identical enzyme or insoluble carrier, and high sensitivity and accuracy can be attained without being affected by undesirable interfering components present in the biological fluid, while no difficult operations are required for the method. In particular,  
115 the method of the present invention permits the highly sensitive determination of antibodies in serum which has been regarded as being difficult in the prior art.

By the way, besides the solid-phase enzyme  
120 immunoassay method, another enzyme immunoassay technique has hitherto been known in which an immunological reaction mixture of immunologically reactive components is subjected to a physicochemical procedure such as gel permeation chromatography, affinity chromatography, or the like to separate the free labeled antigen (or labeled antibody) therefrom, following by measuring the activity of the enzyme contained in the remainder to determine the concentration of antigen or antibody initially present. However, the known enzyme immunoassay  
130

method as above is different in technical concept from the method of the present invention, because the chromatography of the former applies not immunological reaction but physicochemical reaction. Additionally, the method using physicochemical chromatography has, in contrast with the method of the present invention, the disadvantages in that the kinds of labeling enzymes and adsorbing materials for use therein must be carefully selected in particular cases.

#### *Brief description of the drawings*

Figure 1 is a diagrammatic flow sheet illustrating various modes of operation of the immunological reaction step (i.e., the step preceding the enzyme activity measuring step) involved in the method of the present invention;

Figure 2 is a graph illustrating the calibration curve obtained in Example 1 which will be given later; and

Figure 3 is a graph illustrating the calibration curve obtained in Example 5 which will also be given later.

#### *Best modes for carrying out the invention*

Firstly, the immunological reaction step, i.e., the step preceding the enzyme activity measuring step, involved in the method of the present invention can be carried out in various modes of operation. These modes of operation are explained hereinbelow by reference to the diagrammatic flow sheet shown in Figure 1. It is to be understood however that, in the following description, no mention is made of the immunological reaction products containing no labelling enzyme because their presence does not exert any influence on the immunoassay in accordance with the present invention. Where mention is made of a smaller or greater amount of an immunologically reactive component, it means an amount smaller or greater, respectively, than the reaction equivalent to the given amount of the immunologically reactive component to be reacted therewith.

Mode (a): A predetermined amount of a labeled antibody 2-4 is reacted with a smaller amount of an antigen 1 to result an immunological complex 1-2-4 consisting of the labeled antibody 2-4 and of the antigen 1 together with an unbound, free portion 2-4 of the labeled antibody. The reaction mixture is passed through a column packed with a carrier 5 having a greater amount of the antibody 2 adsorbed thereon, whereby an insolubilized product formed by binding of the labeled antibody-antigen complex 1-2-4 to the antibody-on-carrier 5-2 is produced, while the unbound labeled antibody 2-4 is discharged from the column.

Mode (b): Contrary to the mode (a), a predetermined amount of a labeled antigen 1-4 is reacted with a smaller amount of an antibody 2 to result a labeled antigen-antibody complex 2-1-4 and an unbound portion 1-4 of the labeled antigen. The reaction mixture is passed through a column packed with a carrier 5 having a greater amount of an antibody or protein A 3 adsorbed thereon, whereby an insolubilized product is formed by binding of the labeled antigen-antibody complex 2-1-4 to the antibody-on-carrier or protein A-on-carrier 5-3 is produced, while the unbound labeled

antigen 1-4 is discharged from the column.

Mode (c): This mode is the same as the mode (b) above, except that an antigen 1 is added to the reaction system in an amount equivalent to or different from that of the labeled antigen 1-4 (hereinafter referred to briefly a different amount), whereby the immunological reaction is effected to result a labeled antigen-antibody complex 2-1-4 and an unbound, free portion 1-4 of the labeled antigen. The reaction mixture is passed through a column packed with a carrier 5 having a greater amount of an antibody or protein A 3 adsorbed thereon. Thus, an insolubilized product formed by binding of the labeled antigen-antibody complex 2-1-4 to the antibody-on-carrier or protein A-on-carrier 5-3 is produced, while the unbound labeled antigen 1-4 is discharged from the column.

Mode (d): A predetermined amount of labeled antigen 1-4 is mixed with a different amount of an unlabeled antigen 1, and the mixture is passed through a column packed with a carrier 5 having a smaller amount of an antibody 2 adsorbed thereon. Thus, an insolubilized product formed by binding of the labeled antigen 1-4 to the antibody-on-carrier 5-2 is produced, while the unbound labeled antigen 1-4 is discharged from the column.

Mode (e): A predetermined amount of an antigen 1 is passed through a column packed with a carrier 5 having a greater amount of an antibody 2 adsorbed thereon to result antigen-antibody-on-carrier 5-2-1. Subsequently, a greater amount of a labeled antibody 2-4 is passed through the column, whereby an insolubilized product formed by binding of the labeled antibody 2-4 to the antigen-antibody-on-carrier 5-2-1 is produced, while the unbound labeled antibody 2-4 is discharged from the column.

Mode (f): A predetermined amount of an antibody 2 is passed through a column packed with a carrier 5 having a greater amount of an antibody or protein A 3 adsorbed thereon to result antibody-antibody or protein A-on-carrier 5-2-3. Subsequently, a greater amount of a labeled antigen 1-4 is passed through the column, whereby an insolubilized product formed by binding of the labeled antigen 1-4 to the antibody-antibody-on-carrier or antibody-protein A-on-carrier 5-2-3 is produced, while the unbound labeled antigen 1-4 is discharged from the column.

Secondly, according to the method of the present invention, the enzyme activity measuring step following after the immunological reaction step as explained above, is carried out as follows.

After the immunological reaction step has been accomplished by any one of the modes of operation (a)-(f), the column is washed with a buffer solution, and the activity of the labeling enzyme 4 contained in the insolubilized product which has been remained in the column is measured by passing through the column a solution containing a substrate for the enzyme. Typically, the foregoing procedures are previously repeated with varying amounts of the antigen 1 or antibody 2, and the measured values thereby are plotted against the amounts of antigen 1 or antibody 2 initially used to construct a calibration curve. Next, the same procedures as above are

conduct ed by using a biological fluid containing an unknown amount of the antigen 1 or antibody 2. Then, the concentration of the antigen 1 or antibody 2 in the fluid is calculated from the measured value by reference to the calibration curve.

In the practice of the present invention, the immunological reaction, the reaction of an antigen or antibody with a labeling material, and the adsorption of an antibody, an antiantibody, or protein A on an insoluble carrier are attained at a temperature ranging from about 4°C to about 40°C, as is usual with biochemical reactions. In most cases, the time required to complete these reactions is 10 minutes or more.

The antigen 1 to be assayed by the method of the present invention may be any of the hormones and proteins contained in various biological fluids, the functions of some of said proteins being not elucidated. Specific examples of the antigen include insulin, triiodothyronine, thyroxine, calcitonin,  $\alpha$ -fetoprotein, S-100 protein, enolase, calmodulin, secretory immunoglobulin A, and the like. In addition, the blood levels of externally administered drugs, such as antibiotics, can also be determined. The antibody 2 for use in the method of the present invention is an antibody corresponding to the aforesaid antigen 1. Most of these antigens 1, antibodies 2, antiantibodies 3, or biological fluids containing them are commercially available in purified form, and such commercial preparations were used in the examples of the present invention given later.

Specific examples of the labeling enzyme for use in the method of the present invention include  $\beta$ -D-galactosidase, alkaline phosphatase, peroxidase, glucose oxidase, malate dehydrogenase, and the like. Such an enzyme 4 may be coupled to an antigen 1 or an antibody 2 by any conventional procedures. That is, this can be accomplished by using a bifunctional reagent as the coupling agent such as, glutaraldehyde, carbodiimide, N,N'-o-phenylenedimaleimide, m-maleimidobenzoyl-N-hydroxysuccinimide ester, or the like.

Specific examples of the insoluble carrier 5 for use in the method of the present invention include polysaccharides such as agarose, dextran, cellulose, etc.; synthetic resins such as polystyrene, polyacrylamide, etc.; and pieces of glass. In carrying out the method of the present invention, it is desirable that the carrier-adsorbed reactant consisting of an antibody 2, antiantibody 3 or protein A 3 adsorbed on the insoluble carrier 5 is in finely spherical or finely fibrous form, so long as the rate of flow of the immunological reaction mixture through the column is not reduced to an undue extent.

The adsorbing reaction of an antibody 2, antiantibody 3 or protein A 3 on an insoluble carrier 5 may be performed by any conventional procedures. For example, this can be accomplished by activating an insoluble polysaccharide with an activating agent such as cyanogen bromide, sodium periodate, epichlorohydrin, 1,1'-carbonyldiimidazole, p-toluenesulfonyl chloride or the like, followed by adding an antibody or the like to the activated polysaccharide. The amount of antibody 2, antiantibody 3, or protein A 3 for use in this purpose is

suitably in the range of 0.1 to 20 mg per milliliter of the insoluble carrier.

The antibody 2, antiantibody 3 or protein A 3 for use in the method of the present invention may be so-called active fragments isolated therefrom. In the case of an antibody 2 or an antiantibody 3, for example, the active fragments thereof can be isolated by treatment with a protease such as papain, pepsin or the like.

In view of the small sample volumes usually available for immunoassay, of the simplification of operations, and of the attainment of a reasonably short operating time, it is desirable that the capacity of the column for use in the method of the present invention is not greater than 1 ml.

Furthermore, in carrying out the method of the present invention, any interference with the immunological reaction can be prevented by the technique previously proposed by us, that is, by the addition of a hydrophobic protein (e.g., gelatin) together with a salt (e.g., sodium chloride) to the immunological reaction system.

The method according to the present invention is more specifically illustrated by the following examples without however limiting it in any way.

Example 1 Assay of insulin (according to the methods of claims 2 and 6)

(1) Preparation of an antibody and a labeled antibody

A commercial preparation of swine insulin having been extracted from the pancreas of swine was used as the antigen. A commercial preparation of serum containing an antibody having been produced by injecting the antigen into a guinea pig was treated according to conventional procedures, such as fractionation with ammonium sulfate and DEAE-cellulose chromatography, to isolate the antibody therefrom. The antibody thus obtained was split by addition of pepsin and then subjected to column chromatography using Sephadex G-160, whereby the active fragments of the antibody were isolated. These active fragments were reduced by 2-mercaptoethylamine in the usual manner and then reacted with N,N'-o-phenylenedimaleimide to join it to the reduced active fragments. Thereafter, the reaction product was further reacted with a commercial preparation of  $\beta$ -D-galactosidase to prepare the active fragments labeled with  $\beta$ -D-galactosidase (hereinafter referred to simply the labeled antibody).

The aforesaid antigen (i.e., swine insulin) and its corresponding antibody have generally the ability to react immunologically with other serum insulins (as antigens) originated from a wide variety of animal species (including human) and their corresponding antibodies, so that they can be used in the assay of insulins (as antigens) or their corresponding antibodies contained in biological fluids of many animal species (e.g., human).

(2) Preparation of an antibody-on-carrier

An antibody-on-carrier was prepared by adsorbing the antibody obtained in item (1) above on a water-insoluble carrier (CNBr-activated Sepharose) in the usual manner.

(3) Construction of a calibration curve

To 1-ml portions of a buffer solution containing

100  $\mu\text{U/ml}$  of the labeled antibody in the above item (1) were added 0.1 ml each of aqueous solutions containing smaller amounts (i.e., 0 to 320  $\mu\text{U/ml}$ ) of the antigen, and the mixtures were incubated at 37°C for 2 hours to result a labeled antibody-antigen complex together with an unbound portion of the labeled antibody. In this and other examples, "U" represents the unit amount of a hormone having been adopted in the standard bioassay method.

Then, each of the reaction mixtures was passed through a column packed with a greater amount of the antibody-on-carrier prepared in item (2) above, whereby the insolubilized product formed by binding of the labeled antibody-antigen complex to the antibody-on-carrier is produced, while the unbound labeled antibody was discharged from the column. After the column was washed with a buffer solution, 0.1 ml of an aqueous solution containing 10 mg/ml of o-nitrophenyl- $\beta$ -D-galactoside (hereinafter referred to o-NPG) was poured into the column, which was then incubated at 37°C for 2 hours to produce o-nitrophenol. The column was washed with 2 ml of a 0.1 M solution of  $\text{Na}_2\text{CO}_3$  and the absorbance at 420 nm of the filtrate was measured to determine the activity of enzyme having been contained therein. The measured values were plotted against the amounts of antigen initially used to construct a calibration curve as shown in Figure 2 (the method of claim 2).

On the other hand, the above procedure was modified by preparing a series of solutions containing the antigen in the same amounts as before and passing each of these solutions through the column packed with the antibody-on-carrier. After the column was washed, the same amount of the labeled antibody was passed therethrough, followed by measurement of the absorbance of filtrate. In this manner, a calibration curve quite similar to that of Figure 2 was obtained (the method of claim 6).

(4) Determination of insulin in human serum

A human serum sample having unknown concentration (but falling in the range of 0-320  $\mu\text{U/ml}$  as defined in item (3); hereinafter the same) of insulin was used as the antigen, and the same procedure as in the former part of item (3) was carried out. That is, 0.1 ml of the sample was reacted with the labeled antibody, and the reaction mixture was passed through a column packed with the antibody-on-carrier. After o-NPG was poured into the column and incubated, the absorbance of the column filtrate was measured. When calculated from the measured value by reference to the calibration curve shown in Figure 2, the concentration of insulin in the sample was found to be 92  $\mu\text{U/ml}$ . For comparison, the same sample was subjected to a similar procedure in a conventional solid-phase radioimmunoassay (hereinafter referred to RIA) using a radioisotope as the labeling material, so that the concentration of insulin in the sample was estimated to be 102  $\mu\text{U/ml}$ .

On the other hand, the same sample was treated in the same procedure as in the latter part of item (3). Thus, the concentration of insulin in the sample was found to be 95  $\mu\text{U/ml}$ . For comparison, the same sample was subjected to a similar procedure in a conventional RIA, so that the concentration of insulin

the sample was estimated to be 105  $\mu\text{U/ml}$ .

#### Example 2

##### 70 Assay of anti-thyroxine antibody (according to the methods of claims 3 and 7)

###### (1) Preparation of a labeled antigen and an antibody

A commercial preparation of thyroxine having been isolated from an extract of swine thyroid glands was used as the antigen. The amino group of this antigen was coupled with the SH group of  $\beta$ -D-galactosidase in the usual manner to form a labeled antigen. On the other hand, a commercial preparation of serum containing an antibody having been produced by injecting the antigen into a rabbit was immediately used as the antibody.

###### (2) Preparation of an anti-antibody-on-carrier

Generally, immunoglobulin G (hereinafter referred to IgG), which is a kind of protein inherently present in the serum of animals, has the ability to react with antigenic substances originating from a variety of animal species and hence the properties of an antibody corresponding to them. Accordingly, IgG has been used widely for the preparation of an anti-antibody.

In this example, a commercial preparation of serum containing an anti-antibody having been produced by injecting rabbit IgG into a goat was treated in the same manner as in item (1) of Example 1 to isolate the anti-antibody therefrom. By using the anti-antibody so isolated, the same procedure as in item (2) of Example 1 was carried out to prepare an anti-antibody-on-carrier.

###### (3) Construction of a calibration curve

To 0.5-ml portions of a dilution of the labeled antigen prepared in item (1) above were added 3  $\mu\text{l}$  each of serum dilutions containing smaller amounts of the antibody, and after completion of the reaction, each of the reaction mixtures was passed through a column packed with a greater amount of the anti-antibody-on-carrier prepared in item (2). Thereafter, the procedure in item (3) of Example 1 was followed to construct a calibration curve (the method of claim 3).

On the other hand, the above procedure was modified by preparing a series of serum dilutions (0.5 ml each) containing the antibody in the same amounts as before and passing each of the dilutions through a column packed with the anti-antibody-on-carrier. After the column was washed, the same amount of the labeled antigen was passed therethrough, followed by measurement of the absorbance of the column filtrates. In this manner, a quite similar calibration curve to the above-described was obtained (the method of claim 7).

###### (4) Determination of anti-thyroxine antibody in rabbit serum

A rabbit serum sample of unknown concentration of anti-thyroxine antibody was treated in the same procedure as in the former part of item (3) to measure the absorbance of the filtrate. When calculated from the measured value by reference to the calibration curve constructed in item (3) above, the concentration of anti-thyroxine antibody in the sample was found to be 1.05 U/ml. Similarly, the same

sample was treated in the same manner as in the latter part of item (3) and the anti-thyroxine antibody concentration was found to be 1.10 U/ml.

### 5 Example 3

#### *Assay of anti-insulin antibody (according to the method of claim 3)*

##### (1) Preparation of a labeled antigen

- 10 A commercial preparation of swine insulin was used as the antigen. The same procedure as in item (1) of Example 1 was carried out to label the  $\beta$ -D-galactosidase to the antigen. On the other hand, a commercial preparation of serum containing an antibody having been produced by injecting the antigen into a guinea pig was immediately used as the antibody.

##### (2) Preparation of an antiantibody-on-carrier

- 20 A commercial preparation of serum containing an antiantibody (i.e., antibody to guinea pig IgG) having been produced by injecting guinea pig IgG into a rabbit was treated in the usual manner to isolate the antiantibody therefrom. By using the antiantibody, the procedure in item (2) of Example 2 was followed to prepare an antiantibody-on-carrier.

##### (3) Construction of a calibration curve

- To 1-ml portions of a buffer solution containing the labeled antigen prepared in item (1) were added a series of serum dilutions containing smaller amounts of the antibody, and after completion of the reaction, each of the reaction mixtures was passed through a column packed with a greater amount of the antiantibody-on-carrier prepared in item (2). Thereafter, the column was treated in the same procedure as in the former part of item (3) of Example 2 to measure the absorbance of the column filtrate. The measured values were plotted against the amounts of antibody initially used to construct a calibration curve.

- 40 (4) Determination of anti-insulin antibody in guinea pig serum

- A guinea pig serum sample with unknown concentration of anti-insulin antibody was treated in the same procedure as in item (3) above to measure the absorbance of the filtrate. When calculated from the measured value by reference to the calibration curve in item (3) above, the concentration of anti-insulin antibody in the sample was found to be 0.76  $\mu$ U/ml.

### 50 Example 4

#### *Assay of $\alpha$ -fetoprotein (according to the method of claim 2)*

##### (1) Preparation of a labeled antibody

- 55 A commercial preparation of  $\alpha$ -fetoprotein having been isolated from a human serum was used as the antigen. A commercial preparation of serum containing an antibody having been produced by injecting the antigen into a goat was treated in the usual manner to isolate the antibody. By using the antibody, the procedure in item (1) of Example 1 was followed to prepare a labeled antibody.

##### (2) Preparation of an antibody-on-carrier

- By using Toyopearl HW-55 (trademark) as the insoluble carrier, an antibody-on-carrier was pre-

pared in the usual manner.

##### (3) Construction of a calibration curve

- To 1-ml portions of a solution containing the labeled antibody prepared on item (1) were added smaller amounts of the antigen, and after completion of the reaction, each of the reaction mixtures was passed through a column packed with a greater amount of the antibody-on-carrier prepared in item (2). Thereafter, the absorbance of the column filtrate was measured. The measured values were plotted against the amounts of antigen initially used to construct a calibration curve.

##### (4) Determination of $\alpha$ -fetoprotein in human serum

- 80 A human serum sample of unknown concentration of  $\alpha$ -fetoprotein was treated in the same procedure as in item (3) above, to measure the absorbance of the column filtrate. When calculated from the measured value by reference to the calibration curve in item (3) above, the concentration of  $\alpha$ -fetoprotein in the sample was found to be 151 ng/ml. For comparison, the same sample was subjected to a similar procedure in a RIA, and the concentration of  $\alpha$ -fetoprotein in the same was estimated to be 192 ng/ml.

### Example 5

#### *Assay of calcitonin (according to the method of claim 4)*

##### (1) Preparation of an antibody

- A commercial preparation of calcitonin having been isolated from an extract of swine thyroid glands was used as the antigen. A commercial preparation of serum containing an antibody having been produced by injecting the antigen into a rabbit was treated in the same manner as in item (1) of Example 1, to thereby isolate the antibody therefrom.

- 105 (2) Preparation of a labeled antigen and an antiantibody-on-carrier

- The procedure in item (1) of Example 2 was followed to prepare a labeled antigen. On the other hand, an antiantibody-on-carrier was prepared by conducting the same procedure as in item (2) of Example 2.

##### (3) Preparation of antigen solutions having different concentrations

- An aqueous solution of the antigen was diluted to prepare a series of antigen solutions containing 0 to 150 MRC mU/ml of the antigen ("MRC U" refers to the unit amount of calcitonin in the standard bioassay method).

##### (4) Construction of a calibration curve

- 120 To 0.1-ml portions of a solution containing the labeled antigen prepared in item (2) above were added 0.5 ml each of solutions containing smaller amounts of the antibody in item (1) as well as 0.1 ml each of the antigen solutions prepared in item (3). After completion of the reaction each of the reaction mixtures was passed through a column. Thereafter, the column was treated in the same procedure as in item (3) of Example 2 to measure the absorbance of filtrate. The measured values were plotted against the amounts of antigen initially used to construct a

calibration curve as shown in Figure 3.

(5) Determination of calcitonin in a swine thyroid extract

A swine thyroid extract sample having unknown concentration of calcitonin was treated in the same procedure as in item (3) above to measure the absorbance of filtrate. When calculated from the measured value by reference to the calibration curve shown in Figure 3, the concentration of calcitonin in the sample was found to be 75 MRC mU/ml.

#### Example 6

Assay of thyroxine (according to the method of claim 5)

(1) Preparation of a labeled antigen and an antibody

A commercial preparation of thyroxine having been isolated from an extract of swine thyroid glands was used as the antigen. The same procedure as in item (1) of Example 2 was conducted to prepare a labeled antigen. On the other hand, a commercial preparation of serum containing an antibody corresponding to the antigen was treated in the same manner as in item (1) of Example 1 to isolate the antibody therefrom.

(2) Preparation of an antibody-on-carrier

The same procedure as in item (2) of Example 1 was conducted to prepare an antibody-on-carrier.

(3) Preparation of antigen solutions having different concentrations

An aqueous solution of the antigen was diluted to prepare a series of antigen solutions having different concentrations.

(4) Construction of a calibration curve

To 1-ml portions of a solution containing the labeled antigen prepared in item (1) were added the antigen solutions prepared in item (3). Each of the resulting mixtures was passed through a column packed with a smaller amount of the antibody-on-carrier, and the column was treated in the same procedure as in item (3) of Example 1 to measure the absorbance of the column filtrate. The measured values were plotted against the amounts of antigen initially used to construct a calibration curve.

(5) Determination of thyroxine in a swine thyroid extract

A swine thyroid extract sample with unknown concentration of thyroxine was treated in the same procedure as in item (3) above to measure the absorbance of the column filtrate. When calculated from the measured value by reference to the calibration curve constructed in (4) above, the concentration of thyroxine in the sample was found to be 10.5 µg/dl. For comparison, the same sample was subjected to a similar procedure in a RIA and the concentration of thyroxine therein was estimated to be 11.0 µg/dl.

#### 60 CLAIMS

1. A method of solid-phase enzyme immunoassay which comprises the steps of (a) immunological reaction of at least one immunologically reactive component(s) selected from the group consisting of

an antigen, an antibody, a labeled antigen and a labeled antibody, or an immunological complex thereof, with a carrier-adsorbed reactant selected from the group consisting of an antibody-on-carrier, an anti-antibody-on-carrier and protein A-on-carrier at least once, to provide an insolubilized product formed by binding of the labeled antigen (or labeled antibody) or its immunological complex to the carrier-adsorbed reactant together with an unbound, soluble portion of the labeled antigen (or labeled antibody); (b) separating the unbound labeled antigen (or labeled antibody) therefrom; (c) measuring the activity of the enzyme contained in the insolubilized product; (d) constructing by repeating the above steps (a) - (c) a calibration curve representing the relationship between the amount of antigen (or antibody) initially present and the activity of enzyme attached thereto, (e) carrying out the same procedures as above with a biological fluid containing an unknown amount of the antigen (or antibody), to measure the activity of the enzyme contained in the resulting insolubilized product; and (f) calculating the amount (concentration) of antigen (or antibody) present in the biological fluid by reference to the calibration curve, characterized in that all the steps from the step of immunological reaction of the immunologically reactive component(s) or an immunological complex thereof with a carrier-adsorbed reactant (a) through to the step of measuring the activity of enzyme contained in the resulting insolubilized product when the biological fluid has been used (e) are carried out by applying chromatography with a single column.

2. A method as claimed in claim 1 wherein an immunological complex (1-2-4) has been prepared by the reaction of a predetermined amount of a labeled antibody (2-4) with a smaller amount of an antigen (1), while the carrier-adsorbed reactant comprises a greater amount of an antibody (2) adsorbed on a carrier (5).

3. A method as claimed in claim 1 wherein an immunological complex (2-1-4) has been prepared by the reaction of a predetermined amount of a labeled antigen (1-4) with a smaller amount of an antibody (2), while the carrier-adsorbed reactant comprises a greater amount of an anti-antibody or protein A (3) adsorbed on a carrier (5).

4. A method as claimed in claim 1 wherein an immunological complex (2-1-4) has been prepared by the reaction of a predetermined amount of a labeled antigen (1-4) with a smaller amount of an antibody (2) as well as a different amount of an antigen (1), while the carrier-adsorbed reactant comprises a greater amount of an anti-antibody or protein A (3) adsorbed on carrier (5).

5. A method as claimed in claim 1 wherein the immunologically reactive components are a predetermined amount of a labeled antigen (1-4) and a different amount of an antigen (1), while the carrier-adsorbed reactant comprises an antibody (2) adsorbed on a carrier (5).

6. A method as claimed in claim 1 wherein the immunologically reactive components are a predetermined amount of an antigen (1) and a greater amount of a labeled antibody (2-4), and which

immunologically reactive components are passed successively through a column packed with the carrier-adsorbed reactant comprising a greater amount of an antibody (2) adsorbed on a carrier (5).

5 7. A method as claimed in claim 1 wherein the immunologically reactive components are a predetermined amount of an antibody (2) and a greater amount of a labeled antigen (1-4), and which immunologically reactive components are passed successively through a column packed with the carrier-adsorbed reactant comprising a greater amount of an anti-antibody or protein A (3) adsorbed on a carrier (5).

8. A method as claimed in any of claims 2-7 wherein the antigen (1) constituting one of the immunologically reactive components is a member selected from the group consisting of insulin, triiodothyronine, thyroxine, calcitonin,  $\alpha$ -fetoprotein, S-100 protein, enolase, calmodulin and secretory immunoglobulin A.

9. A method as claimed in any of claims 2-7 wherein the insoluble carrier (5) constituting the carrier-adsorbed reactant is a member selected from the group consisting of agarose, dextran, cellulose, polystyrene, polyacrylamide, and pieces of glass.

10. A method as claimed in claim 8 wherein the insoluble carrier (5) constituting the carrier-adsorbed reactant is a member selected from the group consisting of agarose, dextran, cellulose, polystyrene, polyacrylamide, and pieces of glass.

11. A method as claimed in claims any of 2-7 wherein the labeling enzyme (4) constituting the labeled antigen or labeled antibody is a member selected from the group consisting of  $\beta$ -D-galactosidase, alkaline phosphatase, peroxidase, glucose oxidase and malate dehydrogenase.

12. A method as claimed in claim 8 wherein the labeling enzyme (4) constituting the labeled antigen or labeled antibody is a member selected from the group consisting of  $\beta$ -D-galactosidase, alkaline phosphatase, peroxidase, glucose oxidase and malate dehydrogenase.

13. A method as claimed in claim 9 wherein the labeling enzyme (4) constituting the labeled antigen or labeled antibody is a member selected from the group consisting of  $\beta$ -D-galactosidase, alkaline phosphatase, peroxidase, glucose oxidase and malate dehydrogenase.

14. A method as claimed in claim 10 wherein the labeling enzyme (4) constituting the labeled antigen or labeled antibody is a member selected from the group consisting of  $\beta$ -D-galactosidase, alkaline phosphatase, peroxidase, glucose oxidase and malate dehydrogenase.

15. A method as claimed in claim 9, wherein the carrier-adsorbed reactant is in fine spherical or finely fibrous form, such that the rate of flow of the immunological reaction mixture through the column is not reduced to a significant extent.

16. A method as claimed in claim 9, wherein the capacity of the column for use in the chromatography of the present method is not greater than 1 ml.

17. A method as claimed in claim 1 substantially as described in any one of the Examples herein.

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